IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Anne Marie S. Beckerleg, Ph.D.
Art Unit	:	1632
Applicants	:	Richard W. Moyer, Yi Li, Allison L. Bawden
Serial No.	:	09/662,254
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For	:	Materials and Methods for Delivery and Expression of Heterologous DNA in Vertebrate Cells

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF RICHARD W. MOYER, Ph.D., UNDER 37 C.F.R. § 1.132

Sir:

I, Richard W. Moyer, Ph.D., of the University of Florida, hereby declare:

THAT, I am an inventor on the above-referenced patent application;

THAT, I have received the following degrees:

B.S. in Agricultural and Biological Chemistry with Minor in Chemistry; Pennsylvania State University; 1962

Ph.D. in Biochemistry with Minor in Organic Chemistry; University of California at Los Angeles; 1967;

THAT, I have been employed professionally as follows:

1987-present	Professor and Chairman, Department of Immunology and Medical Microbiology, College of Medicine, University of Florida

1981-1987	Professor, Department of Microbiology, Vanderbilt University School of Medicine
1976-1981	Associate Professor, Department of Microbiology, Vanderbilt University School of Medicine
1970-1976	Assistant Professor of Biochemistry, Columbia University
1967-1969	Postdoctoral Fellow, Massachusetts Institute of Technology
6/1965-9/1965	Summer Trainee in Physiology, Woods Hole Marine Biology Laboratory
1963-1967	Research Assistant, Biochemistry, University of California at Los Angeles
1962-1963	Research Assistant, Biochemistry, University of Minnesota

THAT, I have published extensively in my field and some of the publications are as follows:

1. Bawden, A.L., K.J. Glassberg, J. Diggans, R. Shaw, W. Farmerie, R.W. Moyer (2000) "Complete genomic sequence of the *Amsacta moorei* entomopoxvirus: Analysis and comparison with other poxviruses" *Virology* 274(1):120-139;

2. Moon, K.B., P.C. Turner, R.W. Moyer (1999) "SPI-1-dependent host range of rabbitpox virus and complex formation with cathepsin G is associated with serpin motifs" *Journal of Virology* 73(11):8999-9010;

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4. Li, Y., S. Yuan, R.W. Moyer (1998) "The non-permissive infection of insect (gypsy moth) LD-652 cells by Vaccinia virus" *Virology* 248(1):74-82;

5. Li, Y. R.L. Hall, S.L. Yuan, R.W. Moyer (1998) "High-level expression of *Amsacta moorei* entomopoxvirus spheroidin depends on sequences within the gene" *J Gen Virol* 79(Pt 3):613-622;

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11. VanderLeek, M.L., J.A. Feller, G. Sorensen, W. Isaacson, C.L. Adams, D.J. Borde, N. Pfeiffer, T. Tran, R.W. Moyer, E.P.J. Gibbs (1994) "Evaluation of swinepox virus as a vaccine vector in pigs using an Aujeszky's disease (pseudorabies) virus gene insert coding for glycoproteins gp50 and gp63" *The Veterinary Record* 134:13-18;

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14. Martinez Pomares, L., R.J. Stern, R.W. Moyer (1993) "The ps/hr gene (B5R open reading frame homolog) of rabbitpox virus controls pock color, is a component of extracellular enveloped virus, and is secreted into the medium" *Journal of Virology* 67:5450-5462;

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23. Hall, R.L., R.W. Moyer (1991) "Identification, cloning, and sequencing of a fragment of *Amsacta moorei* entomopoxvirus DNA containing the spheroidin gene and three vaccinia related ORFs" *Journal of Virology* 65:6516-6527;

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31. Turner, P.C., R.W. Moyer, eds. (1990) "The Molecular Pathogenesis of Poxviruses," in *The Poxviruses*, Springer Verlag, New York, pp.125-153;

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50. DeLuca, M., K.E. Ebner, D.E. Hultquist, G. Kreil, J.B. Peter, R.W. Moyer, P.D. Boyer (1963) "The isolation and identification of phosphohistidine from mitochondrial protein" *Biochem. Z.* 338:512;

THAT, I am an inventor on the following patents: U.S. Patent No. 5,935,777, "Entomopoxvirus Expression System," issued August 10, 1999; U.S. Patent No. 5,721,352, "Entomopoxvirus Expression System," issued February 24, 1998; U.S. Patent No. 5,651,972, "Use of Recombinant Swine Poxvirus as a Live Vaccine Vector," issued July 29, 1997; U.S. Patent No. 5,476,781, "Entomopoxvirus Spheroidin Gene Sequences," issued December 19, 1995; U.S. Patent 5,212,057, "Biological Systems for Constructing and Testing Viral Vaccines," issued May 18, 1993;

THAT, through my years of research, I have kept up to date on the technical literature and maintained contact with experts in the field by participating in professional meetings and seminars, and by direct personal contact. As a result, I am familiar with the general level of skill of those working in the fields of virology and molecular biology, and in particular the use of viral vectors in genetic engineering.

THAT, I have read and understood the specification and claims of the subject application and the Office Action dated September 4, 2001;

AND, being thus duly qualified, do further declare:

1. The entomopox virus vectors disclosed and claimed in this patent application have been found to be highly effective in delivering polynucleotides to vertebrate cells, resulting in the expression of genes encoded by the polynucleotides.

2. The patent Examiner states that at pages 13-14, bridging paragraph, of the patent application, it is indicated that entomopoxvirus cannot productively infect

mammalian cells and that gene expression is limited to early promoter activity. The patent Examiner also notes that the patent application indicates that late poxvirus promoters, such as AmEPV spheroidin or cowpox virus ATI, are inactive in mammalian cells infected with recombinant EPV. The patent Examiner then concludes "the skilled artisan would not predict that any and all promoter sequences could express a heterologous gene of interest when encoded by a recombinant entomopox virus."

3. The paragraph bridging pages 13-14 of the patent application refers to the general observation that when using entomopoxvirus vectors containing entomopox promoters, genes under the control of early entomopox promoters will be expressed in a vertebrate cell, but genes under the control of late entomopox promoters will not. The same paragraph of the patent application refers to two publications (Li *et al.* [1997] and Gauthier *et al.* [1995]), both of which describe experiments using entomopox virus vectors containing entomopox promoters. However, as explained below, the distinction between "early" promoters and "late" promoters only finds context with respect to poxvirus promoters (vertebrate and insect poxvirus promoters).

4. When insect poxviruses infect vertebrate cells, early and only early poxvirus promoters are active. This is likely because the early poxvirus transcription apparatus is packaged within the virion particle as part of creating virions from the previous infection. In vertebrate cells, following early promoter-driven expression, the infection then aborts and eventually the input virus particles disintegrate, after which the viral DNA is released into the vertebrate cell's cytoplasm; hence, the lack of late poxvirus promoter-driven expression in vertebrate cells. This observation is also made within the Li *et al.* publication (1997) at page 9557, second column, page 9560, second column, and page 9561, second column, which is cited by the patent Examiner. As indicated within the Li

et al. publication, at page 9561, second paragraph, and at page 71, lines 3-7, of the patent application, an exception to this phenomenon is when cells infected with EPV vectors containing late poxvirus promoters are supplied with certain additional factors *in trans* (*e.g.*, by co-infection with vaccinia virus), which at least partially rescue late gene expression through activation of late promoters.

5. However, as taught within the patent application, early poxvirus promoters and (under certain circumstances) late poxvirus promoters are not the only promoters that can be used in an entomopoxvirus vector to achieve expression of a foreign gene within a vertebrate cell. Entomopoxvirus vectors containing genes under the control of non-poxvirus promoters can also be utilized, such as vertebrate host cell promoters and promoters from other viruses. This is documented at page 75, lines 11-24, and page 81, lines 15-24, of the patent application. Specifically, non-poxvirus promoters that are recognized by the vertebrate host cell's nuclear RNA polymerase, such as the cytomegalovirus (CMV) and herpes TK gene promoters, can be used following entomopoxvirus infection, entry of the viral DNA into the nucleus, and selection for stable transformation of the vertebrate host cell. Once transformed, those foreign genes that are under the control of the non-poxvirus promoters within the nuclear environment become activated, leading to expression of the foreign gene. Preferred promoters are those constitutive or regulatable promoters, such as the CMV or Herpes TK gene promoters, capable of promoting sufficient expression of the foreign DNA contained within the viral vector in a vertebrate cell.

6. The patent Examiner states that Example 11 of the patent application only examines expression of β-galactosidase expression at day two, following injection, "and does not correlate the level or duration of gene expression with any therapeutic effect." However, it would be expected that expression of the

foreign gene, β-galactosidase in this case, would be sustained for a period beyond the two days at which time the tissue was excised.

7. The patent Examiner states the skilled artisan would not predict that the entomopox virus vectors of the invention could be used to express therapeutic levels of protein in lymphoid cells, which are associated with certain disorders, such as Burkitt's lymphoma. Although it has been reported in the literature that there is a greater level of expression in some cells (*e.g.*, fibroblasts) than in other cells, such as lymphoid cells, one would expect that greater levels of expression could be achieved, for example, through the use of tissue-specific promoters and/or *in vitro* selection steps.

8. In summary, given the teachings of the patent application, one would expect that the entomopoxvirus vectors of the invention can be used to deliver and express foreign genes within a vertebrate cell, using a variety of promoters.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed: Robert W. Moyer

Richard W. Moyer, Ph.D.

Date: January 11, 2002

Field: **Author,** Limits: **Publication Date from 1998 to 1998**

1: Gene Ther. 1998 Dec;5(12):1656-64.

A strategy for enhancing the transcriptional activity of weak cell type-specific promoters.

Nettelbeck DM, Jerome V, Muller R.

Institut fur Molekularbiologie und Tumorforschung (IMT), Philipps-Universitat Marburg, Germany.

Cell type- and tissue-specific promoters play an important role in the development of site-selective vectors for gene therapy. A large number of highly specific promoters has been described, but their applicability is often hampered by their inefficient transcriptional activity. In this study, we describe a new strategy for enhancing the activity of weak promoters without loss of specificity. The basic principle of this strategy is to establish a positive feedback loop which is initiated by transcription from a cell type-specific promoter. This was achieved by using a cell type-specific promoter to drive the simultaneous expression of the desired effector/reporter gene product and a strong artificial transcriptional activator which stimulates transcription through appropriate binding sites in the promoter. Using a VP16-LexA chimeric transcription factor, we show that this approach leads to a 14- to > 100-fold enhancement of both the endothelial cell-specific von Willebrand factor promoter and the gastrointestinal-specific sucrase-isomaltase promoter while maintaining approximately 30- to > 100-fold cell type specificity.

PMID: 10023445 [PubMed - indexed for MEDLINE]

Dec 6 2004 18:18:14

1: Hum Gene Ther. 1997 Nov 20;8(17):2019-29.

Comment in:

- Hum Gene Ther. 1997 Nov 20;8(17):2009-10.

Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression.

Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS.

Department of Surgery, University of Michigan, Ann Arbor 48109-0331, USA.

One of the major limitations to current gene therapy is the low-level and transient vector gene expression due to poorly defined mechanisms, possibly including promoter attenuation or extinction. Because the application of gene therapy vectors in vivo induces cytokine production through specific or nonspecific immune responses, we hypothesized that cytokine-mediated signals may alter vector gene expression. Our data indicate that the cytokines interferon-gamma (IFN-gamma) and tumor necrosis factor-alpha (TNF-alpha) inhibit transgene expression from certain widely used viral promoters/enhancers (cytomegalovirus, Rous sarcoma virus, simian virus 40, Moloney murine leukemia virus long terminal repeat) delivered by adenoviral, retroviral or plasmid vectors in vitro. A constitutive cellular promoter (beta-actin) is less sensitive to these cytokine effects. Inhibition is at the mRNA level and cytokines do not cause vector DNA degradation, inhibit total cellular protein synthesis, or kill infected/transfected cells. Administration of neutralizing anti-IFN-gamma monoclonal antibody results in enhanced transgene expression in vivo. Thus, standard gene therapy vectors in current use may be improved by altering cytokine-responsive regulatory elements. Determination of the mechanisms involved in cytokine-regulated vector gene expression may improve the understanding of the cellular disposition of vectors for gene transfer and gene therapy.

PMID: 9414251 [PubMed - indexed for MEDLINE]

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Field: **Title/Abstract,** Limits: **Publication Date from 1995 to 1998**

1: Hum Gene Ther. 1998 Mar 1;9(4):477-85.

Transient immunosuppression allows transgene expression following readministration of adeno-associated viral vectors.

Manning WC, Zhou S, Bland MP, Escobedo JA, Dwarki V.

Chiron Corporation, Emeryville, CA 94608, USA.

Adeno-associated viral (AAV) vectors have much promise in gene therapy. Among the many properties that make AAV an ideal vector for gene therapy are its ability to infect both dividing and nondividing cells and the longevity of expression in tissues such as brain, skeletal muscle, and liver. However, like other viral vectors, readministration of vector is limited because of the host's immune response to viral components of the vector. Using class I, class II, and CD40 ligand (CD40L)-deficient mice, we demonstrate that neutralizing antibodies to the viral capsid proteins prevent transgene expression following readministration of rAAV vectors. Transient immunosuppression of mice by treatment with antibody to CD4 at the time of primary infection allowed transgene expression after readministration of rAAV vectors to animals. Transient immunosuppression with antibody to CD40L had only a modest effect on the efficacy of readministration. The ability to readminister virus was inversely correlated with both AAV capsid enzyme-linked immunosorbent assay titers and AAV neutralizing antibody titers. These studies demonstrate that readministration of rAAV can be accomplished by down regulating the anti-AAV immune response and suggest the use of repeated administration of rAAV as a viable form of therapy for the treatment of chronic diseases.

PMID: 9525309 [PubMed - indexed for MEDLINE]

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Oct 29 2004 13:56:18

Constitutive Expression of phVEGF$_{165}$ After Intramuscular Gene Transfer Promotes Collateral Vessel Development in Patients With Critical Limb Ischemia

Iris Baumgartner, MD; Ann Pieczek, RN; Orit Manor, PhD; Richard Blair, MD; Marianne Kearney, BS; Kenneth Walsh, PhD; Jeffrey M. Isner, MD

Background—Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries, a concept called "therapeutic angiogenesis." The objectives of this phase 1 clinical trial were (1) to document the safety and feasibility of intramuscular gene transfer by use of naked plasmid DNA encoding an endothelial cell mitogen and (2) to analyze potential therapeutic benefits in patients with critical limb ischemia.

Methods and Results—Gene transfer was performed in 10 limbs of 9 patients with nonhealing ischemic ulcers (n=7/10) and/or rest pain (n=10/10) due to peripheral arterial disease. A total dose of 4000 μg of naked plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor (phVEGF$_{165}$) was injected directly into the muscles of the ischemic limb. Gene expression was documented by a transient increase in serum levels of VEGF monitored by ELISA. The ankle-brachial index improved significantly (0.33±0.05 to 0.48±0.03, P=.02); newly visible collateral blood vessels were directly documented by contrast angiography in 7 limbs; and magnetic resonance angiography showed qualitative evidence of improved distal flow in 8 limbs. Ischemic ulcers healed or markedly improved in 4 of 7 limbs, including successful limb salvage in 3 patients recommended for below-knee amputation. Tissue specimens obtained from an amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells by immunohistochemistry. PCR and Southern blot analyses indicated persistence of small amounts of plasmid DNA. Complications were limited to transient lower-extremity edema in 6 patients, consistent with VEGF enhancement of vascular permeability.

Conclusions—These findings may be cautiously interpreted to indicate that intramuscular injection of naked plasmid DNA achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in selected patients with critical limb ischemia. (***Circulation.*** **1998;97:1114-1123.)**

Key Words: angiogenesis ■ genes ■ ischemia ■ growth substances

Critical limb ischemia is estimated to develop in ≈500 to 1000 individuals per million per year.[1] In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization, and the disease thus frequently follows an inexorable downhill course.[2,3] Psychological testing of such patients has disclosed quality-of-life indices similar to those of patients with cancer in the terminal phase of their illness.[4] As concluded in the Consensus Document of the European Working Group on Critical Limb Ischemia,[1] no pharmacological treatment has been shown to favorably affect the natural history of critical limb ischemia.[5] Indeed, amputation, despite its associated morbidity, mortality, and functional implications,[1,6-8] is often recommended as a solution to the disabling symptoms, in particular excruciating ischemic rest pain, of critical limb ischemia.[9-12] A second major amputation will be required in nearly 10% of such patients. Despite the use of prosthetics and rehabilitation, reestablishment of full mobility is inconsistently achieved, particularly in the elderly. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling.

See p 1108

Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral[13,14] and myocardial[15-17] ischemia, a concept called therapeutic angiogenesis. Several of these studies have used VEGF, also known as vascular permeability factor, a secreted endothelial-cell mitogen with high-affinity binding sites limited to endothelial cells.[18-22] Endothelial cell specificity has been considered to represent an important advantage of VEGF for therapeutic angiogenesis, because endothelial cells represent the critical cellular element responsible for new vessel formation.[23-25]

Received October 9, 1997; revision received December 22, 1997; accepted January 13, 1998.

From the Departments of Medicine (Cardiology) (I.B., A.P., J.M.I.), Radiology (R.B.), and Biomedical Research (I.B., O.M., M.K., K.W., J.M.I.), St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Mass.

Correspondence to Jeffrey M. Isner, MD, St Elizabeth's Medical Center of Boston, 736 Cambridge St, Boston, MA 02135.

E-mail jisner@opal.tufts.edu

Selected Abbreviations and Acronyms

ABI = ankle-brachial index
MRA = magnetic resonance angiography
PCR = polymerase chain reaction
$phVEGF_{165}$ = plasmid encoding 165-amino-acid isoform of human VEGF
TBI = toe-brachial index
VEGF = vascular endothelial growth factor

We recently demonstrated angiographic and histological evidence of angiogenesis after intra-arterial gene transfer of naked plasmid DNA encoding human VEGF in a patient with critical limb ischemia.[26] In this report, we present the results of intramuscular $phVEGF_{165}$ gene transfer performed in an initial phase 1 clinical trial comprising 9 patients with 10 critically ischemic limbs.

Methods

Patients

Patients qualified for intramuscular gene therapy if they (1) had chronic critical limb ischemia[1] including rest pain and/or nonhealing ischemic ulcers present for a minimum of 4 weeks without evidence of improvement in response to conventional therapies and (2) were not optimal candidates for surgical or percutaneous revascularization.[27] Requisite hemodynamic deficit included a resting ABI <0.6 and/or TBI <0.3 in the affected limb on 2 consecutive examinations performed at least 1 week apart. Criteria used to describe a change in limb status were adapted from standards recommended by the Society for Vascular Surgery/North American Chapter and International Society for Cardiovascular Surgery.[3,28] Patients were allowed to continue on aspirin and coumarin, provided that these therapies had been used for a minimum of 6 months before gene transfer. Vasoactive medications were discontinued unless prescribed for cardiac disease or systemic hypertension. All patients gave written informed consent for their participation. The study was designed as a phase 1, nonrandomized study to document the safety of intramuscular $phVEGF_{165}$ gene transfer and to monitor patients as well for evidence of bioactivity. This study design was unanimously approved by the Recombinant DNA Advisory Committee of the National Institutes of Health, by the Human Institutional Review Board and Institutional Biosafety Committee of St Elizabeth's Medical Center, and by the US Food and Drug Administration.

Plasmid DNA ($phVEGF_{165}$)

All patients received a eukaryotic expression vector encoding the $VEGF_{165}$ gene[29] transcriptionally regulated by the cytomegalovirus promoter/enhancer.[26] Preparation and purification of the plasmid from cultures of $phVEGF_{165}$-transformed *Escherichia coli* were performed in the Human Gene Therapy Laboratory at St Elizabeth's Medical Center by the column method (Qiagen Mega Kit, Qiagen, Inc). The purified plasmid was stored in vials and pooled for quality control analyses.

Intramuscular $phVEGF_{165}$ Transfer

Aliquots of 500 μg of $VEGF_{165}$ pDNA were diluted in sterile saline, and 4 aliquots (total, 2000 μg) were administered into calf and/or distal thigh muscles of the patients by direct intramuscular injection into the ischemic limb. The injection sites were arbitrarily selected according to available muscle mass and included sites above as well as below the knee. The volume of each of the 4 injectates per limb was progressively increased during the course of the study from 0.75 mL (3 treatments) to 3 mL (6 treatments) to 5 mL (11 treatments). Four weeks after the first 2000-μg injection, a second 2000-μg injection was administered, increasing the total amount of pDNA to 4000 μg per patient. One patient was treated for bilateral critical limb ischemia with a total amount of 8000 μg pDNA (4000 μg per limb).

Serum VEGF Levels

ELISAs were performed at baseline and weekly up to 12 weeks after the initial treatment of 7 limbs to detect evidence of gene expression at the protein level. Samples were immediately centrifuged for 20 minutes at 3600 rpm at 4°C, and the serum was stored at −20°C until analysis. Serum VEGF was determined with an immunoassay according to the manufacturer's instructions (R&D Systems). Results were compared with a standard curve of human VEGF with a lower detection limit of 5 pg/mL. Samples were checked by serial dilution and were performed at least in duplicate.

Hemodynamic and Angiographic Assessment

Patients were followed up on a weekly basis within the first 8 weeks after gene therapy and at monthly intervals thereafter. Ischemic ulcers were documented by color photography. Resting ABI and TBI were calculated by the quotient of absolute ankle or toe pressure to brachial pressure.[30] Intra-arterial digital subtraction angiography and MRA were performed within 1 week before and 4 weeks after each treatment and 3 months after the latter of 2 intramuscular injections. Digital subtraction angiography was performed as a selective single-leg runoff study using undiluted nonionic contrast media (Isovue-370, Squibb Diagnostics). A minimum of 2 images (early and late frames) at the thigh, knee, calf, and ankle/foot levels were recorded by digital acquisition and hard copies in a 35×45-cm format. The diameter of newly visible collateral vessels was assessed by comparison with a 0.09-in-diameter reference wire taped to the skin. MRA was performed with a 1.0-T superconducting system (Impact, Siemens) by means of a transmit-receive extremity coil, a body coil, or both and commercially available pulse sequences. A multisection two-dimensional time-of-flight gradient echo sequence without intravenous contrast medium was used.[31] All axial images were reconstructed by use of the maximum-pixel-intensity algorithm at intervals of 60°.

Immunohistochemistry

Double immunohistochemical staining for proliferating endothelial cells was performed as previously described.[32] Bound antibody was then detected with an alkaline phosphatase kit (Biogenex Laboratories). Complexes were visualized with fast red substrate (Biogenex Laboratories). A counterstain of 10% Gill hematoxylin was applied before coverslips were applied.

DNA Analysis

Skin specimens at the site of gene injection and muscle specimens near or remote from the site of gene injection were retrieved from 2 amputees 8 and 10 weeks after intramuscular $phVEGF_{165}$ transfer, respectively (patients 4 and 10, Table). Tissue was processed with a genomic DNA isolation kit (A.S.A.P., Boehringer Mannheim). For PCR analysis, primer sets unique to the promoter and VEGF coding region of $phVEGF_{165}$ were selected. For Southern analysis, *Eco*R1-digested total cellular DNA (30 μg) and purified $phVEGF_{165}$ DNA (0.5 μg) were subjected to 0.8% agarose electrophoresis. The predicted sizes of *Eco*R1-digested plasmid fragments were 998 and 4703 bp. DNA blotted to a nylon membrane (Amersham, Life Science) was hybridized with two ^{32}P-labeled $phVEGF_{165}$-specific probes (*nco*I-digested 679-bp $phVEGF_{165}$ fragment, position 389 to 1068; *ava*I-digested 787-bp $phVEGF_{165}$ fragment, position 991 to 1778), washed, and exposed to Hyperfilm MP (Amersham, Life Science).

Statistical Analysis

Data are reported as mean±SEM. Comparisons between paired variables were performed with the nonparametric Friedman test and Wilcoxon rank sum test. All statistical tests were two-tailed, with a significance level of $P<.05$.

Clinical, Hemodynamic, Angiographic, Laboratory, and Molecular Findings Before and After Intramuscular phVEGF$_{165}$ Gene Transfer

			Clinical History and Findings Before Gene Therapy				Outcome After Gene Transfer	
No.	Sex	Age, y	Cigs, pk/y	DM	Previous Treatment	Signs/Symptoms	Limb Status	DSA Findings
1	F	33†	30	0	4 bypass grafts, 3 rev., prostaglandins	Calf ulcer, toe gangrene (digit I)	ABI +0.24; complete healing→limb salvage	New collaterals, 200–400 μm
2	F	53	0	+	3 bypass-grafts, 1 PTA, prostaglandins	Toe gangrene (digit V)	ABI +0.12; complete healing	New collaterals, 200–400 μm
3	M	77	0	+	None	Toe gangrene (digits I, IV)	TBI +0.11; gangrene/ osteomyelitis→BKA	New collaterals, 200–400 μm
4	F	39†	20	0	Sympathectomy	Forefoot gangrene	ABI +0.27; forefoot necrosis→BKA	New collaterals, 200–400 μm
5	M	74	90	0	1 PTA	Rest pain	ABI +0.15; rest pain resolved	New collaterals, 200–800 μm
6	F	84	40	0	6 bypass grafts, 1 PTA	Toe gangrene (digits I–V)	ABI +0.22; toe amputation→limb salvage	None
7	F	80	20	0	1 bypass graft	Rest pain	ABI unchanged, rest pain resolved	New collaterals, 200–800 μm
8*	F	39	20	0	Sympathectomy	Heel ulcer, toe gangrene (digits I–IV)	ABI +0.22; toe amputation→limb salvage	New collaterals, 200–>800 μm
9	M	54	30	0	4 bypass grafts, 2 rev., 1 PTA	Rest pain	TBI +0.18; rest pain resolved	None
10	M	54	70	0	6 bypass-grafts, 1 PTA	Toe gangrene (digits I, III, IV)	No change in ABI/TBI, BKA	None

No. indicates consecutively treated ischemic limbs; Cigs, current cigarette smoker; pk/y, pack years of cigarette smoking; DM, diabetes mellitus (non–insulin-dependent DM, oral medication); DSA, digital subtraction angiography; BI, baseline; rev., surgical revisions; PTA, percutaneous transluminal angioplasty; TBI (ABI incompressible); BKA, below-knee amputation; ND, not done; and pos., positive.

*No. 8 and 4 identical patient (bilateral treatment).

†Suspected Buerger's disease (stopped smoking >3 months before study entry).

Results

Demographic and clinical data for the 5 women and 4 men (mean age, 59±19 years) treated with phVEGF$_{165}$ are shown in the Table. Average length of follow-up at the time of this report was 6±3 months (range, 2 to 11 months). Local intramuscular gene transfer induced no or mild local discomfort up to 72 hours after the injection. Serial creatine phosphokinase measurements remained in the normal range, there were no signs of systemic or local inflammatory reactions, and no patient developed antibodies to VEGF. To date, neither loss of visual acuity nor change in funduscopic examination due to diabetic retinopathy[33] has been observed in any patient treated with phVEGF$_{165}$ gene transfer. Likewise, no development of a latent neoplasm[34] has been observed. The only complication seen was transient lower-extremity edema, consistent with VEGF enhancement of vascular permeability.[35]

Transgene Expression

Blood levels of VEGF transiently peaked 1 to 3 weeks after gene transfer in 7 patients in whom weekly blood samples were obtained (Fig 1). (In 2 patients, baseline and/or more than two follow-up blood samples were not obtained.) Indirect clinical evidence of VEGF overexpression was evident from the development of peripheral edema (+1 to +4 by gross inspection) in the 6 patients with ischemic ulcers. In 4 of these patients, edema was limited to the treated limb, whereas in 2 patients, the contralateral limb was affected as well, albeit less severely. The onset of edema corresponded temporally to the rise in serum VEGF levels.

Noninvasive Arterial Testing

Absolute systolic ankle or toe pressure increased in 8 limbs after gene transfer and was unchanged in 1 limb at the time of the most recent follow-up (53±4.8 at baseline, 66±4.6 most recent follow-up, $P=.008$). ABI and/or TBI increased from 0.33±0.05 (range, 0 to 0.58; n=10) at baseline to 0.43±0.04 (0.22 to 0.57, $P=.028$; n=10) at 4 weeks, to 0.45±0.04 (0.27 to 0.59, $P=.016$; n=10) at 8 weeks, and to 0.48±0.03 (0.27 to 0.67, $P=.017$; n=8) at 12 weeks (Fig 2). Improvement in the pressure index was sustained, but the increases in values obtained after the second (4-week) injection were not significantly different from measurements made 4 weeks after the initial injection. Exercise performance improved in all 5 patients with rest pain or ischemic ulcers who were able to perform graded treadmill exercise.[36] All patients experienced a significant increase in pain-free walking time (2.5±1.1 minutes before gene therapy versus 3.8±1.5 minutes at an average of 13 weeks after gene therapy, $P=.043$). A statistically significant increase in absolute, claudication-limited

Continued

VEGF Level, pg/mL			
BI	1st	2nd	Molecular Findings
47	223	607	
36	ND	ND	
46	131	780	
30	59	888	PCR pos. in skin + muscle specimens; Southern pos. in muscle specimens
62	300	96	
29	164	80	
40	44	223	
ND	ND	ND	
0	113	83	
ND	ND	ND	PCR pos. in skin + muscle specimens; Southern pos. in muscle specimens

walking time (4.2±2.1 minutes before versus 6.7±2.9 minutes after gene therapy, $P=.018$) was documented as well. Two patients reached the target end point of 10 minutes of exercise.

Angiography

Digital subtraction angiography showed newly visible collateral vessels at the knee, calf, and ankle levels in 7 of 10 treated ischemic limbs. The luminal diameter of the newly visible vessels ranged from 200 to >800 μm, although most were closer to 200 μm; the latter frequently appeared as a "blush" of innumerable collaterals (Fig 3A and 3B). Follow-up angiograms disclosed no evidence of collateral artery regression in any patients. Serial magnetic resonance angiograms of the ischemic limb disclosed qualitative evidence of improved distal blood flow in 8 limbs, including enhancement of signal intensity in previously identified vessels, and an increase in the number of newly visible collaterals (Fig 3C and 3D).

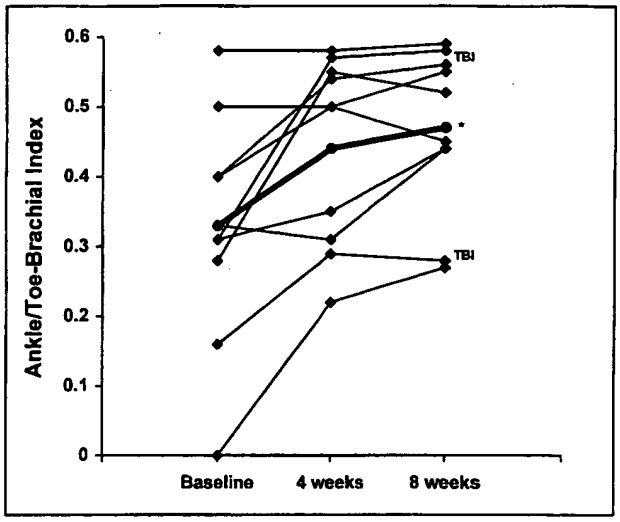


Figure 2. Gain in ABI and/or TBI in 10 limbs 4 and 8 weeks after intramuscular phVEGF$_{165}$ gene transfer. *Mean values, $P=.02$.

Change in Limb Status and Ischemic Rest Pain

Therapeutic benefit was demonstrated by regression of rest pain and/or improved tissue integrity in the ischemic limb. Limb salvage, for example, was achieved in a 33-year-old woman (patient 1, Table), who had undergone 7 unsuccessful surgical reconstructions at another hospital. She presented with a necrotic great toe and a 9×3-cm ischemic ulcer at the site of vein harvest in her distal left limb (Fig 4). The ulcer had failed to respond to 6 months of conservative measures, during the last 3 of which she had been treated with methadone, oxycodone/acetaminophen, amitriptyline hydrochloride, and a fentanyl patch. She had been advised by her vascular surgeons to undergo below-knee amputation. Within 8 weeks after gene transfer, her ABI had increased by 0.24, and the ulcer dimensions had diminished sufficiently to permit placement of a split-thickness skin graft. The graft healed successfully and remained healed at 9-month fol-

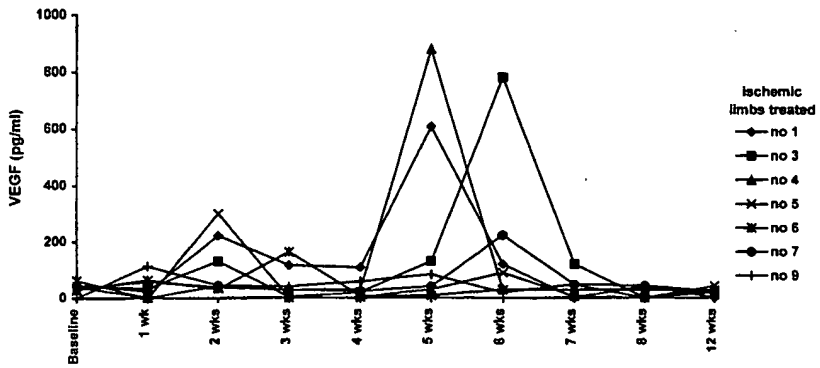


Figure 1. Serial levels of VEGF determined by ELISA disclosed a transient elevation 1 to 2 weeks after intramuscular (phVEGF$_{165}$) gene transfer. Baseline and/or weekly follow-up venous blood samples, which were incomplete in 3 of 10 treated limbs (patients 2, 8, and 10 in the Table), are not shown.

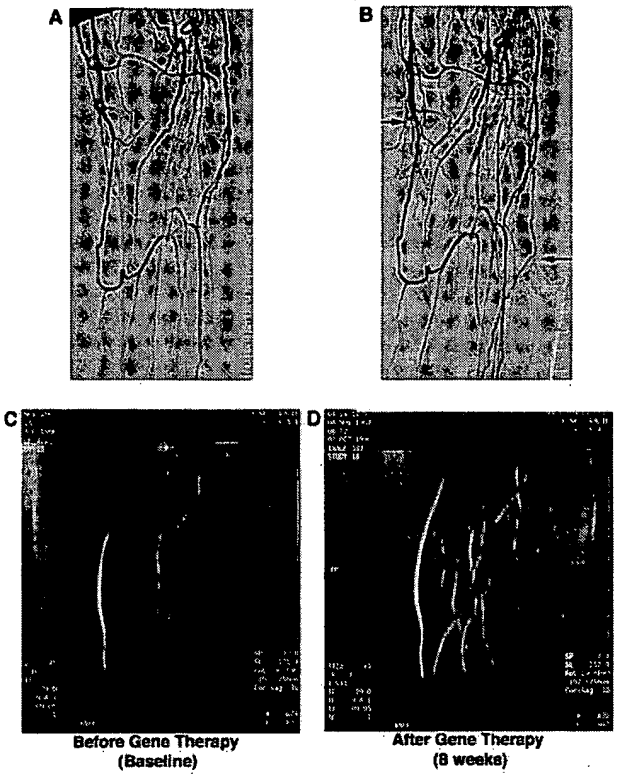


Figure 3. A and B, Newly visible collateral vessels at calf level 8 weeks after phVEGF$_{165}$ gene transfer. Luminal diameter of newly visible vessels ranged from 200 to >800 μm (arrow); most were closer to 200 μm, and these frequently appeared as a blush of innumerable collaterals. C and D, MRA before and 8 weeks after gene therapy. After gene therapy, signal enhancement is clearly evident, consistent with improved flow in ischemic limb.

low-up (Fig 4). A second patient, a 39-year-old woman (patients 4 and 8, Table), presented with a 3-month history of gangrene of the distal half of her right foot. Although the ABI in her right limb improved by 0.27 after gene transfer, the forefoot gangrene was not reversed, and she underwent right below-knee amputation. While she was being treated for the right limb, however, she developed gangrene in the left limb (Fig 5). After gene transfer to the left limb, the ABI in her left lower extremity increased by 0.22 in association with angiographically demonstrable, newly visible collateral vessels. Although she required amputation of the left great toe, the operative site healed promptly, and her remaining 4 toes and heel recovered completely, including restoration of normal nail growth (Fig 5). In 2 other patients, complete (patient 2, Table) or partial (patient 6, Table) healing of ischemic ulcers present for 12 and 2 months, respectively, avoided major limb amputation. In the 3 patients (patients 5, 7, and 9, Table) who presented with rest pain (of 6, 5, and 3 months' duration, respectively) unassociated with loss of tissue integrity, rest pain resolved completely in all 3 patients after gene transfer and has remained so at the most recent follow-up.

For the total group of 10 limbs, frequency of ischemic rest pain expressed as afflicted nights per week decreased significantly (5.9±2.1 at baseline versus 1.5±2.8 at 8-week follow-up, P=.043). On the basis of criteria proposed by Rutherford et al,[3] limb status improved in 9 of 10 extremities treated (Table). Moderate improvement, including both an upward shift in clinical category (≥1 clinical category in patients with rest pain and ≥2 categories in patients with tissue loss) and an increase of >0.1 in the ABI, was documented in 5 patients.

Immunohistochemistry and Molecular Analyses

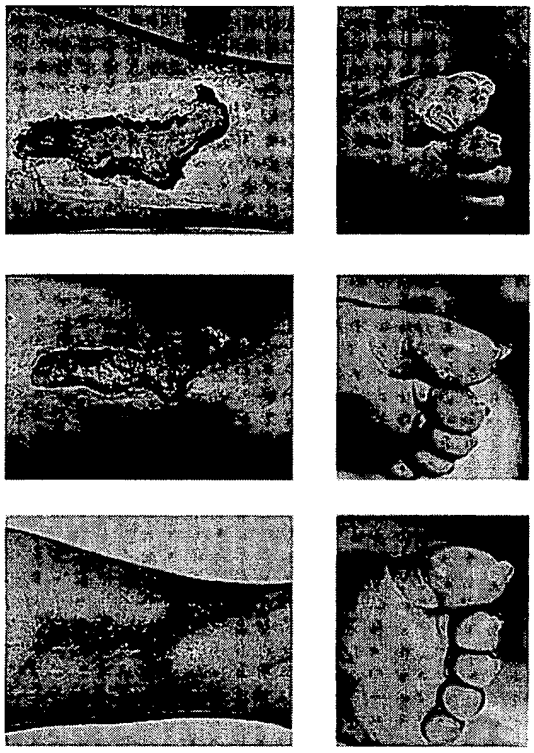
Tissue specimens retrieved from 1 amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells (Fig 6A). This finding was particularly striking given that endothelial cell proliferation is nearly absent in normal arteries.[37] PCR performed on these samples indicated persistence and anatomic redistribution of DNA fragments unique to phVEGF$_{165}$. Noteworthy amplification of DNA fragments was shown in muscle and skin samples derived from the site of injection as well as in several muscle samples remote from the injection site (Fig 6B). Southern blot analysis confirmed persistence of intact plasmid DNA in muscle specimens derived from 2 amputees 8 and 10 weeks after gene therapy (patients 4 and 10, Table) (Fig 6C).

Discussion

The natural history of critical limb ischemia has been well documented to have an inexorable downhill course.[3] The inclusion criteria for this study were drafted to restrict treatment to patients in whom the natural history of critical limb ischemia had been established previously. Seven of the 10 limbs had developed frank gangrene. Although inclusion criteria required a minimum of 4 weeks of conservative measures without evidence of improvement, in reality, signs and/or symptoms of critical limb ischemia had been present in all cases for 2 to 12 months before gene therapy. Among this series of 9 patients (10 limbs), 6 developed critical limb ischemia despite having undergone as many as 7 vascular surgical reconstructions. Seven patients had been specifically advised to undergo limb amputation. All were using analgesic, typically ≥1 narcotic, medications. Spontaneous resolution of rest pain and/or healing of an ischemic ulcer in patients like these with critical limb ischemia has not to our knowledge been reported previously.[1] Furthermore, because VEGF had not been administered previously as recombinant protein, no data were available from any source to indicate either the safety or bioactivity of any dose of phVEGF$_{165}$. Accordingly, the design of this phase 1 trial, unanimously approved by the Recombinant DNA Advisory Committee and the US Food and Drug Administration, was conducted as a nonrandomized, consecutive treatment series, similar to phase 1 oncology protocols used to study new chemotherapeutic agents administered to human subjects.

Analysis of gene expression at the protein level by use of an ELISA assay for VEGF documented a transient peak of the gene product in the systemic circulation 1 to 3 weeks after gene transfer in 7 patients. Further evidence of gene expression was observed in 6 patients, who developed temporally related peripheral edema, including 2 with bilateral edema. Parenthetically, the latter finding constitutes what is to the best of our knowledge the first demonstration that VEGF may augment vascular permeability in human subjects.

In most patients, treatment was sufficient to achieve clinically significant modulation of the recipient phenotype.



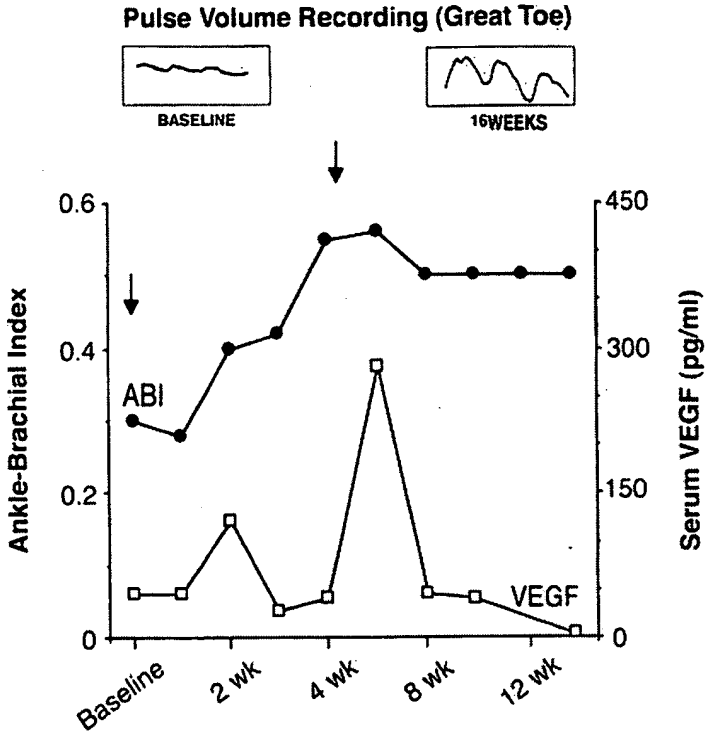


Figure 4. Limb salvage after gene therapy in a 33-year-old woman (patient 1, Table). Left top, Nonhealing wound on medial aspect of calf and ischemic necrosis involving great toe. Left middle, Ingrowth of granulation tissue in calf wound, healing of great toe. Left bottom, Three months after gene transfer, healing of split-thickness skin graft at wound site and full resolution of great toe necrosis. Before gene therapy, patient was wheelchair-bound on multiple analgesics, including methadone, amitriptyline hydrochloride, clonidine, oxycodone/acetaminophen, and a fentanyl patch. Three months after gene transfer, she was freely ambulatory and had been successfully weaned from all analgesics. Right, Evidence of $phVEGF_{165}$ bioavailability documented by an increase in venous VEGF blood levels and bioactivity expressed as an increase in ABI. The ABI progressively increased from 0.28 before to 0.56 after gene therapy (weeks refer to time after transfection). This was associated with development of a phasic pulse volume recording compared with nonphasic tracing recorded at baseline. Vertical arrows indicate timing of each of the 2 intramuscular $phVEGF_{165}$ injections.

Noninvasive studies documented hemodynamic evidence of improved limb perfusion that satisfies outcome criteria proposed to assess the results of surgical reconstruction or percutaneous revascularization.[28] Absolute ankle and/or toe pressure increased in 8 limbs after gene therapy ($P=.008$). ABI and/or TBI increased from 0.33 ± 0.05 at baseline to 0.48 ± 0.03 at 12 weeks ($P=.017$). To put this in perspective, an increase of >0.1 in the ABI is considered indicative of a successful surgical or percutaneous intervention.[28] To the best of our knowledge, such improvement has not previously been achieved spontaneously or with medical therapy in patients with critical limb ischemia.

Similarly, angiographic demonstration of newly visible collateral vessels, accompanied here by noninvasive (MRA) evidence of improved blood flow, has to the best of our knowledge not been reported previously in response to any therapeutic intervention. Indeed, previous reports have indicated that current methods used to perform diagnostic contrast angiography cannot provide images of arteries measuring <200 μm in diameter[38]; the spatial resolution of images obtained by MRA is even less. Using synchrotron radiation microangiography to assess collateral artery development after VEGF gene transfer in a rat model of hindlimb ischemia, Takeshita et al[38] showed that neovascularization included a substantial contribution of vessels <180 μm in diameter. Thus, conventional angiographic techniques used in the present study may have failed to depict the full extent of angiogenesis achieved after $phVEGF_{165}$ transfection, particularly given that most newly visible collaterals were diminutive (200 to 800 μm).

That angiogenesis was in fact therapeutic in the present investigation was shown by concomitant reduction in rest pain and/or a favorable impact on limb integrity. Rest pain resolved in all 3 of the patients who presented with rest pain alone. Ischemic ulcers present in 7 limbs healed or improved markedly in 4 patients; this included 3 patients recommended for below-knee amputation in whom successful limb salvage was achieved. Given the poor prognosis for patients with chronic critical limb ischemia, in whom the possibility of spontaneous improvement is remote,[1,2] the outcome in this initial cohort is thus encouraging.

Beginning with the reports of Wolff et al,[39] work from several laboratories[40-48] convincingly demonstrated evidence of transgene expression after direct injection of nonviral, covalently closed pDNA into skeletal muscle. The conceptual basis for therapeutic angiogenesis after $phVEGF_{165}$ gene transfer in particular has been established previously by our laboratory.[49,50] The results of the present trial extend previous findings from studies performed in live animals[46] to patients with advanced peripheral artery disease.

The failure of previous gene therapy trials to yield evidence of clinical success has been attributed to gene delivery,

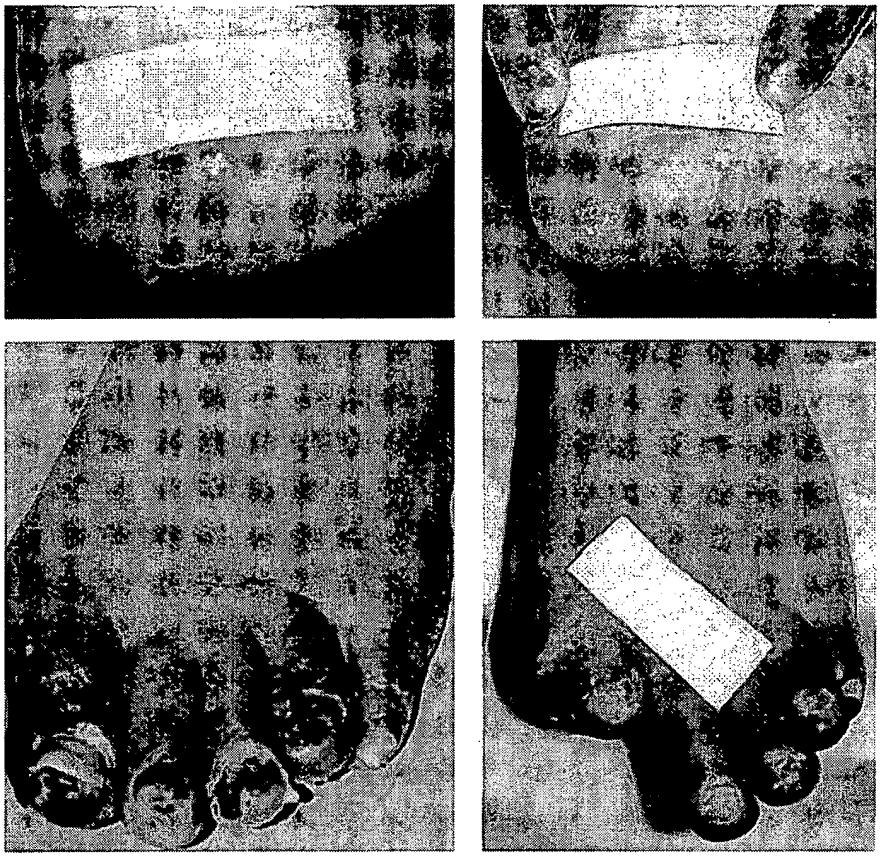


Figure 5. Limb salvage after gene therapy in a 39-year-old woman (patient 8 and 4, Table). This patient presented with a 3-month history of gangrene of distal one-half of right foot. Although ABI in right limb improved by 0.27, forefoot gangrene was not reversed, and she underwent below-knee amputation. While she was being treated for right limb, however, she developed gangrene of left great toe and shortly after, of 4 remaining left toes as well. After gene transfer to left limb, her ABI increased by 0.22 in association with angiographically demonstrable new collateral vessels. Although she required a great toe amputation, operative site healed promptly, and remaining 4 toes recovered completely, including restoration of normal nail growth.

specifically the inability to deliver genes efficiently and to obtain sustained expression.[51] Those cases in which phVEGF$_{165}$ gene therapy led to successful clinical outcomes in this clinical trial suggest that the success of gene therapy is not solely a function of transfection efficiency, nor is it necessarily dependent on protracted gene expression. Several aspects of the gene, protein, and target tissue may have contributed to successful modulation of the host phenotype, despite the relatively low transfection efficiency typically associated with naked DNA. First, VEGF, as noted above, is actively secreted by intact cells; previous studies in our laboratory[52] have documented that genes that encode for secreted proteins, as opposed to proteins that remain intracellular, may yield meaningful biological outcomes because of paracrine effects of the secreted gene product. Second, heparin avidity of the VEGF$_{165}$ isoform promotes binding to cell surface and matrix heparan sulfates that may create a biological reservoir of the secreted protein, enhancing the temporal opportunity for bioactivity. Third, although endothelial cells were previously viewed solely as the target for VEGF, it is now clear that endothelial cells subjected to hypoxia can synthesize VEGF as well.[53] This autocrine feature of VEGF creates the opportunity for amplifying the effects of even a small amount of exogenous VEGF, because endothelial cell proliferation in the ischemic territory creates additional potential cellular sources of VEGF synthesis and secretion. Fourth, VEGF inhibits apoptosis,[54] in part by upregulating endothelial cell expression of fibronectin and $\alpha_v\beta_3$,[54,55] thus preserving the survival signal generated by attachment of endothelial cells to their extracellular matrix. Such reduction in endothelial cell apoptosis would be expected to complement the mitogenic effect of VEGF, resulting in a further net increase in endothelial cell viability. Fifth, with regard to the target of gene therapy, it has been noted[14,26,49] that VEGF-induced angiogenesis is not indiscriminate or widespread but rather is restricted to sites of ischemia. This appears to result from paracrine upregulation of the principal high-affinity VEGF receptor (*Kdr*) in response to factors released from hypoxic skeletal myocytes.[56] Receptor upregulation on endothelial cells within the region of lower-limb or myocardial ischemia thus enables these cells to act as magnets for any VEGF secreted into the ischemic milieu. Finally, the fact that the host tissues are by definition hypoxic may directly aid intramuscular transfer of naked DNA, because transfection efficiency is augmented when the injected skeletal muscle is ischemic.[40,46]

Previous work from our laboratory established that phVEGF$_{165}$ transgene expression is limited to <30 days in animal models of limb ischemia.[26,46,49] Although Southern blot and PCR analyses indicated that small amounts of plasmid DNA were preserved in tissue specimens derived from 2 amputees in this clinical trial, we have no evidence to suggest that transgene expression is more protracted in human subjects than in our animal models. Fortuitously, however, it appears that in both animals and humans, collateral vessel development sufficient to restore limb perfusion to satisfactory resting levels occurs within this time interval. Cessation of gene expression beyond this time point can be considered to constitute an inherent safety feature of phVEGF$_{165}$ gene

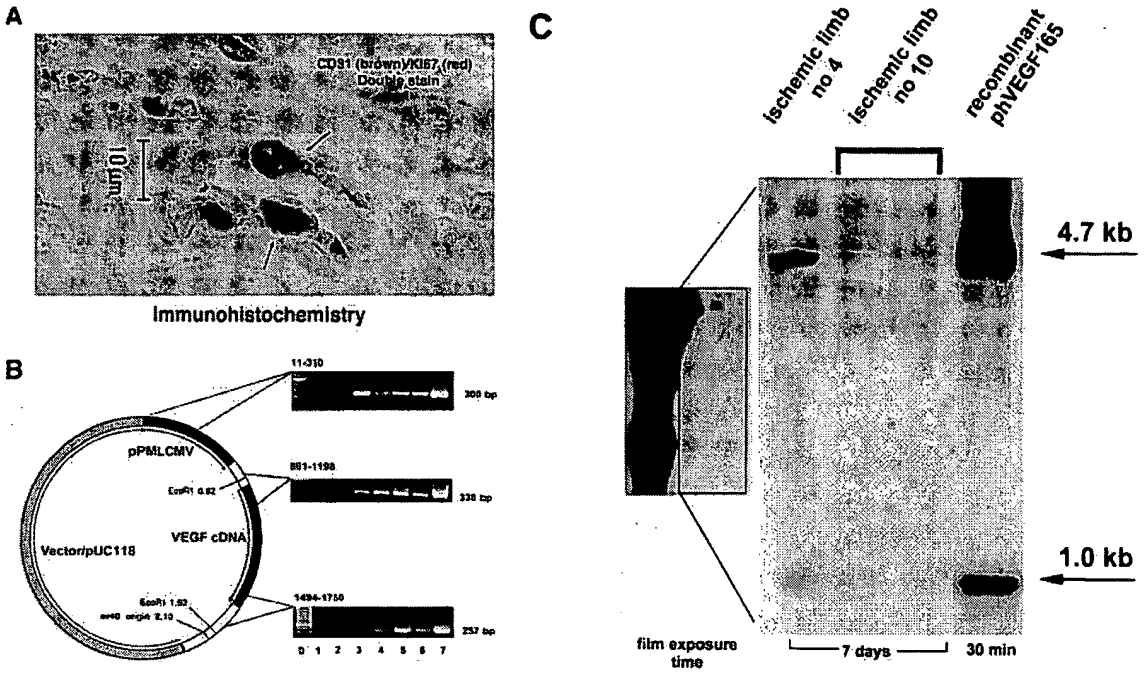


Figure 6. Immunohistochemical and molecular analyses of tissue specimens derived from 2 amputees 8 and 10 weeks after gene therapy (limbs 4 and 10, Table). A, Double immunohistochemical staining of tissue specimen with monoclonal antibody to CD31 (brown) and polyclonal antibody to Ki-67 (red) shows proliferating microvascular endothelial cells (arrows). B, PCR demonstrates amplification of $phVEGF_{165}$ DNA fragments in skin and skeletal muscle specimens. Lane 0 shows 100-bp ladder; lane 2, reaction mixture without tissue DNA; lane 3, negative control (skin specimen from untreated patient); lanes 4 to 6, specimens derived from $phVEGF_{165}$-treated amputee (patient 4, Table); lane 4, skin; lane 5, gastrocnemius muscle (remote from site of transfection); lane 6, tibialis anterior muscle (transfection site); lane 7, positive control (purified $phVEGF_{165}$). Amplified fragments had predicted sizes of 300, 338, and 257 bp, spanning the CMV promoter/enhancer region (5′-CCCGACATTGATTATTGA-3′ and 5′-CGGGCCATTTACCGTCAT-3′; position 11 to 300), proximal VEGF encoding region and junction between VEGF encoding region and promoter (5′-GCCTTTCTCTCCACAGGT-3′ and 5′-GTACTCGATCTCATCAGG-3′; position 861 to 1198), and distal VEGF encoding region and junction between VEGF encoding region and SV40 polyadenylation sequence (5′-CGCGTTGCAAGGCGAGGC-3′ and 5′-GGACCCAAAGTGCTCTGC-3′; position 1494 to 1750), respectively. C, Southern blot analysis of *Eco*R1-digested total cellular DNA (30 µg) (lane 1, patient 4, Table; lanes 2 and 3, patient 10, Table), and 0.5 µg (lane 4) of purified $phVEGF_{165}$ DNA hybridized with two ^{32}P-labeled $phVEGF_{165}$ specific probes (position 389 to 1068 and position 991 to 1778) showed persistence of complete plasmid DNA (*Eco*R1-digested 4703- and 998-bp fragments) in skeletal muscle specimens derived from 2 amputees (patients 4 and 10, Table).

transfer that protects the organism from indefinite constitutive expression of an angiogenic growth factor.

Several caveats regarding this preliminary clinical experience must be acknowledged. First, it is theoretically possible that VEGF expression resulting from gene transfer could promote the development of a tumor that is currently too small to be recognized. Previous laboratory studies, however, have established that VEGF expression, although sufficient to promote a growth process, did not lead to malignant proliferation or to metastasis, a finding in agreement with the notion that stimulation of angiogenesis is necessary but not sufficient for malignant growth.[34,57] It is also theoretically possible that VEGF may aggravate deteriorating eyesight due to diabetic retinopathy.[33] To date, however, no change in visual acuity has been observed in any patient treated with $phVEGF_{165}$ gene transfer. Nevertheless, these findings are preliminary and do not establish the long-term safety of VEGF, administered either as a gene or gene product. Second, although it is conceivable that continuous, predominantly local production of VEGF resulting from the transgene may be preferable, from the standpoints of both safety and efficacy, to a single larger dose of recombinant protein, this notion remains to be proven. Preliminary clinical trials of recombinant VEGF protein therapy have confirmed that mild hypotension seen in preclinical studies[15,58] may be seen in humans as well (unpublished data). Presumably, the route and/or dose of recombinant protein delivery can be adjusted to address this issue. Clearly, further clinical studies of both recombinant protein and alternative dosing regimens of gene therapy will be required to define the relative merits of each approach. Third, we cannot exclude the possibility that these encouraging preliminary results might have been made more substantial and/or uniform by the use of alternative vector systems and/or dosing strategies.[45,48,51,59]

In summary, these preliminary data may be cautiously interpreted to support both the strategy of intramuscular gene therapy and the concept of therapeutic angiogenesis for treatment of selected patients with critical limb ischemia.

Acknowledgments

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